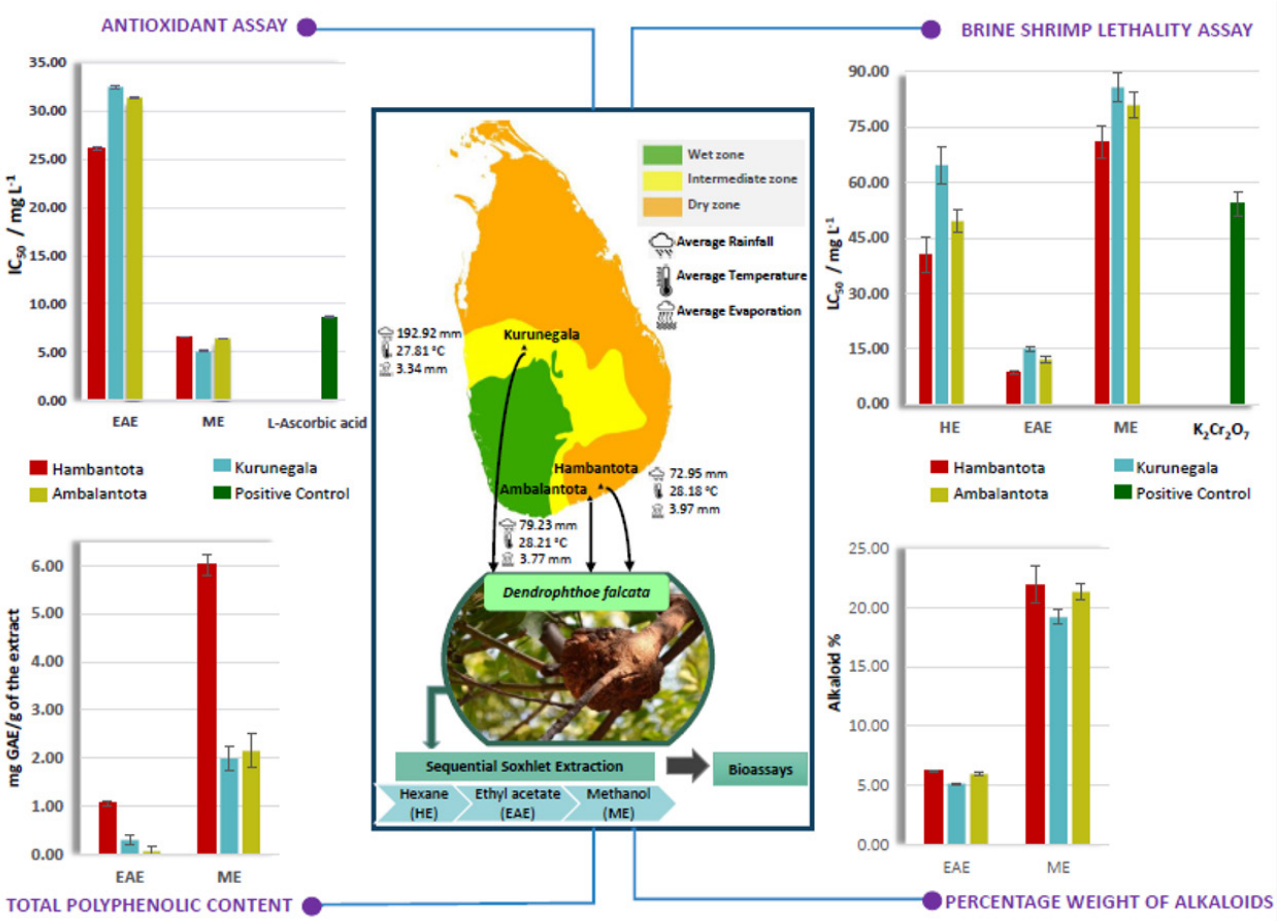
RESEARCH ARTICLE

Impact of environmental regions in Sri Lanka on the bioactivity of *Dendrophthoe falcata* grown on the host *Limonia acidissima*

S. M. N. Anjalee and D. R. Uduwela*



Highlights

- Hambantota belonging to dry zone has the most stressful environmental conditions from the three locations.
- Total polyphenolic content of Hamb-ME shows 180-200% higher activity compared to other locations.
- Brine shrimp lethality of all extracts of Hambantota shows relatively higher toxicity.
- Bioactivity is influenced by environmental stress which reflects higher production of secondary metabolites.

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Impact of environmental regions in Sri Lanka on the bioactivity of *Dendrophthoe falcata* grown on the host *Limonia acidissima*

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Abstract: *Dendrophthoe falcata* (L.f.) Ettingsh (Loranthaceae family) is a hemiparasite which has many medicinal applications and grows on a variety of hosts. Bioactivity of the hemiparasite shows a great host dependence, where it contains high antioxidant activity coupled with high toxicity when grown on the host *Limonia acidissima*. This study aimed to investigate the impact of environmental conditions on the bioactivity of the hemiparasite grown on *L. acidissima* (Rutaceae family), as the environmental stress may play a determining role in the production of secondary metabolites in a plant. The hemiparasite grown in Hambantota (Hamb – dry zone), Kurunegala (Kuru – intermediate zone) and Ambalantota (Amba – dry zone), were selected for this study. Sequential extracts by Soxhlet method from hexane (HE), ethyl acetate (EAE) and methanol (ME) were compared for antioxidant activity, polyphenolic content, toxicity and alkaloid content determined by DPPH, Folin-Ciocalteu, brine shrimp lethality and acid-base assays respectively. Antioxidant activity was approximately 40% higher for Kuru-ME compared to Hamb-ME and Amba-ME. A significant 180 - 200% higher polyphenolic content was observed for Hamb-ME compared to the Amba-ME and Kuru-ME. Toxicity studies revealed that Hamb-EAE is 43% and 29% more toxic than Kuru-EAE and Amba-EAE. The alkaloid content of Hamb-ME showed the highest percentage with a less significant difference between the extracts of other two locations. On average, among the three locations, extracts of Kurunegala, Ambalantota and Hambantota showed the least, intermediate and highest bioactivities respectively experiencing the least, intermediate and most environmental stressed conditions. Hence, it can be concluded that the bioactivity is influenced by the environmental stress due to the impact of governing the secondary metabolites produced by *Dendrophthoe falcata*.

Keywords: Bioactivities; *Dendrophthoe falcata*; environmental stress; secondary metabolite production.

INTRODUCTION

Nature bears the capability of producing new potential therapeutic compounds due to its tremendous species diversity that results in a vast chemical variety. Out of the millions of species of plants, animals, marine organisms and microorganisms, almost all bear biosynthetic compounds comprising medicinal values (Rocha *et al*., 2001).

Dendrophthoe falcata (L.f.) Ettingsh is one of the large branched hemiparasitic plants that belongs to the Loranthaceae family of mistletoes. Several plant hosts are reported for the hemiparasites such as *Mangifera indica, Calotropis gigantean, Tamarindus indicus, Shorea robusta, Artocarpus heterophyllus, Swietenia fabrilis* and *Limonia acidissima* (Dashora *et al*., 2011a; Haque *et al*., 2014; Priya *et al*., 2016; Karunaratne and Uduwela, 2020) The genus *Dendrophthoe* comprises about 31 species spread across tropical Africa, Asia, and Australia. It is indigenous to Sri Lanka, India, China, Thailand, Australia, Malaysia, Myanmar and Bangladesh. *D. falcata* is reported for numerous medicinal applications where aerial parts, leaves and bark of *D. falcata* contain several biologically active compounds representing antioxidant activity, anti-diabetic activity, cytotoxicity, antitumor activity, wound healing ability and antifertility activity (Pattanayak *et al*., 2008; Dashora *et al*., 2011a, 2011b). Further, the bioactivity of the hemiparasite has proven to show a great dependence on the host it grows on (Karunaratne and Uduwela, 2020).

In addition to the host dependence of the hemiparasite, the production of secondary metabolites in a plant can vary due to internal factors such as plant parts and biochemical factors as well as external factors such as geographical location, climate, nature of the soil, salinity, wind, radiation, pollutants, heavy metals, pesticides, season and growth conditions (Pavarini *et al*., 2012; Sampaio *et al*., 2016). Several studies have reported the increased production of secondary metabolites upon the exposure to greater environmental stress. This is due to the external pressure acting on the plant that can affect the secondary metabolic pathways and trigger the production of secondary metabolites to prepare the plant species to face adverse environmental conditions to ensure its survival. (Arumugam *et al*., 2015; Sampaio *et al*., 2016). This study focuses on assessing the variation of bioactivity of *D. falcata* grown on *Limonia acidissima* (wood apple) in three locations belonging to different environmental regions of Sri Lanka as each region experiences comparatively diverse environmental stress, which could have an effect on the production of secondary metabolites and in turn affect the bioactivity.

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MATERIALS AND METHODS

Plant specimen collection, identification and extraction

The stem of *D. falcata* on host *Limonia acidissima* was obtained from three different locations: Kurunegala (Kuru), Ambalantota (Amba) and Hambantota (Hamb). The collected plant specimens were authenticated by the national herbarium at the Royal Botanical Gardens, Peradeniya. Stems of *D. falcata* were thoroughly washed, cut into small pieces, air dried for two weeks, and ground to a fine powder. Sequential extraction was performed by the Soxhlet apparatus using hexane (Hamb-HE, Kuru-HE, Amba-HE), ethyl acetate (Hamb-EAE, Kuru-EAE, Amba-EAE) and methanol (Hamb-ME, Kuru-ME, Amba-ME).

Determination of antioxidant activity

The DPPH assay was used to determine the antioxidant activity as described by Dashora *et al*., (2011a) with slight modifications. Briefly, a concentration series was prepared in methanol for *D. falcata* extracts and L-ascorbic acid as the positive control (Table 1). The initial absorbance of each solution in quartz cuvettes was recorded at 517 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800). Then, DPPH solution in methanol (1 mM, 200 µL) was added to each solution and was kept in the dark for 15 minutes. The absorbance of each solution was remeasured at 517 nm. A negative control was prepared by mixing 200 µL of the DPPH solution with 3.00 mL of methanol and the absorbance was measured. Finally, the percentage antioxidant activity was calculated and the IC_{50} values were determined. All experiments were carried out in triplicate.

Determination of total polyphenolic content

Total polyphenolic content was determined by Folin-Ciocalteu method as described by Meda *et al*., (2005) with slight modifications. First, 60 mg L^{-1} stock solutions in 70% (v/v) methanol were prepared for the *D. falcata* extracts. The final concentration of the extract in the cuvette was kept to 6 mg L^{-1} after the addition of other reagents. The extract solutions (400 µL each) were mixed with 10% Folin-Ciocalteu solution (2.00 mL) in separate test tubes. After 8 minutes 7.5% (w/v) Na_2CO_3 (1.60 mL) was added to each test tube and was left for 1 hour at room temperature. Then the absorbance was measured at 765 nm using the UV-Vis spectrophotometer. A calibration plot for gallic acid was constructed using a concentration series (1, 2, 4, 6, 8, 10 and 20 mg L^{-1}) following the same procedure. The total polyphenolic content was expressed as gallic acid equivalent. All experiments were carried out in triplicate.

Determination of brine shrimp lethality

Brine shrimp lethality assay was conducted as a preliminary determination of toxicity according to a method described by Sarah *et al*., (2017). A concentration series for *D. falcata* extracts (1, 10, 20, 40, 60, 80, 100 mg L^{-1}) and $K_2Cr_2O_7$ as the positive control (1, 5, 50, 125, 250, 500, 1000 mg L^{-1}) was prepared by dissolving in artificial seawater with 1% (v/v) DMSO (Toutianoush *et al*., 2005). Each solution in the concentration series (4.00 mL) was transferred to separate test tubes and ten numbers of the 48-hour old brine shrimp nauplii were added. These test tubes were kept at room temperature for 24 hours and the number of surviving nauplii was counted in each test tube. Artificial sea water with 1% (v/v) DMSO was used as the negative control. The LC_{50} values were determined using probit analysis of Minitab software. All experiments were carried out in triplicate.

Estimation of alkaloid percentage

The acid-base method was followed to determine the percentage of alkaloids in *D. falcata* extracts (Sharma *et al*., 2010). First, the extracts (1.000 g) were dissolved in the respective solvents (40.00 mL) they were extracted from. Then, HCl (40.00 mL) followed by DCM (40.00 mL) were added and the mixtures were shaken in separatory funnels. The aqueous layer was separated and NaOH (2 mol dm^{-3}) was added dropwise until just basic. Then alkaloids were extracted into DCM (40.00 mL) using a separatory funnel and rotary evaporated to obtain a solid crude. Finally, the weight of the alkaloid crude was measured and expressed as a weight percentage. This procedure was carried out in triplicate.

Measuring bark pH of the host tree

Chips (20.000 g, 2 × 2 × 2 cm^3) of *L. acidissima* bark of the three host trees were placed in glass beakers and 50 mL of de-ionized water was added. After 8 hours, samples were filtered and the pH was determined using a pH meter (ORION 420A) (Grodzińska, 1979; Herk *et al*., 2001). All experiments were carried out in triplicate.

Climatic data

Climatic data for Hambantota (dry zone), Kurunegala (intermediate zone) and Ambalantota (dry zone) were

Table 1: Concentration series prepared for DPPH assay.

Sample	Concentration series / mg L^{-1}
Hexane extracts (HE)	50, 100, 200, 300, 400, 500, 1000
Ethyl acetate extracts (EAE)	5, 10, 15, 20, 30, 40, 50
Methanol extracts (ME)	0.5, 1, 2, 4, 6, 8, 10
L-Ascorbic acid (positive control)	2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20

obtained from the Department of Meteorology, Colombo for the years 2017 and 2018 as the sample collection was done at the beginning of 2019.

Statistical analysis

The results of the bioactivity assays were statistically analyzed by considering the 95% confidence intervals of the differences of mean replicates measured between the same extracts of different locations using R-studio software (version 1.4). If the null hypothesis is rejected the difference of means is assumed significant while it is assumed not significant if otherwise. Null (H_0) and alternative (H_1) hypotheses for the test are defined as follows: H_0: $\mu_i = \mu_j$ and H_1: $\mu_i \neq \mu_j$ for $i \neq j$ and i, j = 1,2,3, where μ_i and μ_j are means of the assay results of different samples.

RESULTS AND DISCUSSION

The host tree, *L. acidissima*, is mainly grown in the intermediate and dry climatic zones in Sri Lanka and rarely observed in the wet climatic zone (Ratnayake *et al*., 2020). Hence, sample collection for this study was done from Kurunegala (North Western Province - intermediate zone), Ambalantota and Hambantota (Southern Province – dry zone) (Figure 1) and the wet climatic zone was not considered.

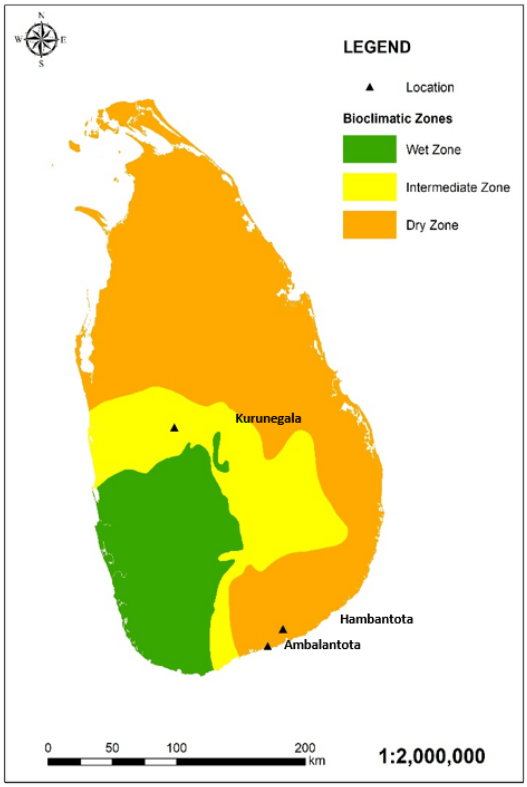

Figure 1: Locations of sample collection: Hambantota, Kurunegala and Ambalantota.

Collection of adequate amount of samples from the Northern Sri Lanka belonging to the dry bioclimatic zone was not successful due to the poor or no growth of the hemiparasite on the host in those regions. The discussion on the differences in bioactivity results is based on the statistical analysis performed by R-studio software.

The antioxidant activity of the *D. falcata* extracts exhibits slight variations between the extracts of different locations indicated by the IC_{50} values determined by the DPPH assay (Figure 2).

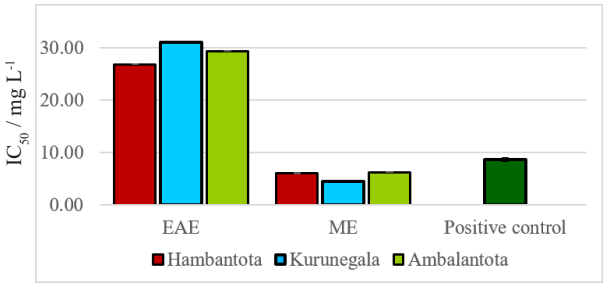

Figure 2: Comparison of IC_{50} values of antioxidant activity.

Antioxidant activity of hexane extracts was remarkably low (IC_{50} > 500 mg L^{-1}) when compared to ethyl acetate and methanol extracts, hence they were not considered here. Methanol extracts showed four-seven-fold greater antioxidant activity compared to the respective ethyl acetate extracts as well as, they showed lower IC_{50} values compared to the positive control, L-ascorbic acid. Among the methanol extracts, the highest antioxidant activity was observed in Kuru-ME with an IC_{50} value of 4.49 mg L^{-1} (Dashora *et al*., 2011a; Pattanayak *et al*., 2012). There is no significant difference between the IC_{50} values of Hamb-ME and Amba-ME and are approximately 40% greater than that of Kuru-ME. Among the ethyl acetate extracts, the lowest antioxidant activity was observed for Kuru-EAE with an IC_{50} value of 31.05 mg L^{-1}. The IC_{50} value of Hamb-EAE is 13% lower than Kuru-EAE while Amba-EAE gave an intermediate activity. These results suggest that the antioxidant activity of *D. falcata* has a dependence on the location of plant growth with a variance observed between the same extracts of different locations except the methanol extracts from Hambantota and Ambalantota.

The Folin-Ciocalteu method was used to determine the total polyphenolic content of the ethyl acetate and methanol extracts of *D. falcata*. Due to the correlation between polyphenolic content and antioxidant activity, the hexane extracts which exhibited remarkably low antioxidant activity were not screened for the total polyphenolic content (Alonso *et al*., 2002; Choi *et al*., 2007; Tawaha *et al*., 2007). Total polyphenolic content was expressed as gallic acid equivalent (GAE) obtained from the standard calibration curve.

The highest polyphenolic content was observed for methanol extracts, correlating well with the antioxidant activity (Figure 3). Hamb-ME exhibited the highest polyphenolic content which was approximately 200% and 180% higher than that of Kuru-ME and Amba-ME respectively. Out of the ethyl acetate extracts, Hamb-EAE showed the greatest polyphenolic content which was followed by Kuru-EAE and Amba-EAE respectively. When considering all three locations, the extracts of Hambantota bear the highest amount of polyphenolic compounds, depicting a significant dependence on the location of plant growth. The correlation of antioxidant

activity and polyphenolic content between the methanol extracts of different locations seemed to be slightly altered as Kuru-ME showed the greatest antioxidant activity followed by Hamb-ME and Amba-ME, whereas the Hamb-ME showed the greatest polyphenolic content followed by Amba-ME and Kuru-ME. This suggests that there could be other compounds in addition to polyphenolic compounds extracted into methanol in different amounts that can act as potent antioxidants (Velioglu *et al*.,1998; Schwarz *et al*., 2009).

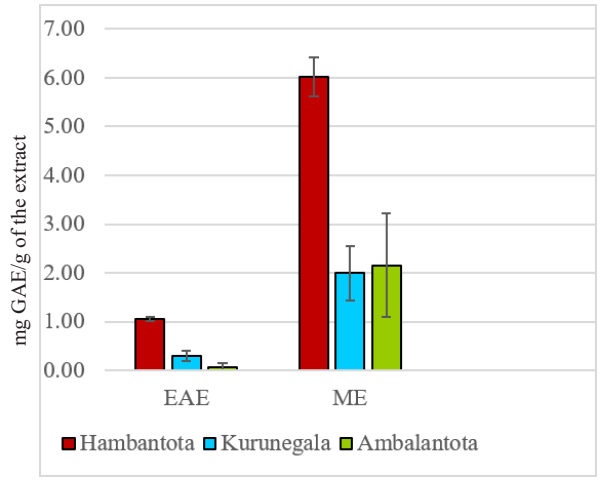

Figure 3: Comparison of total polyphenolic content.

Toxicity of the *D. falcata* extracts was investigated by brine shrimp lethality and expressed as LC_{50} values. The ethyl acetate extracts exhibited the highest brine shrimp lethality, while methanol and hexane extracts exhibited the lowest and intermediate activities respectively which aligned well with reported literature (Karunaratne and Uduwela, 2020). When compared across the regions, the extracts from Hambantota showed the greatest toxicity while the extracts from Kurunegala and Ambalantota showed the least and intermediate toxicities respectively in all three solvent extracts. Hamb-EAE showed the greatest toxicity out of the ethyl acetate extracts with 43% and 29% greater toxicity than Kuru-EAE and Amba-EAE respectively (Figure 4).

It is worth noting that the ethyl acetate extracts showed greater toxicity than the positive control, $K_2Cr_2O_7$. These results indicate that the plant specimen collected from Hambantota location has a higher production of toxic compounds compared to the other two locations. Percentage of alkaloids present in *D. falcata* was determined by acid-base method. The methanol extracts contain approximately four-fold greater alkaloid content compared to the respective ethyl acetate extracts (Figure 5).

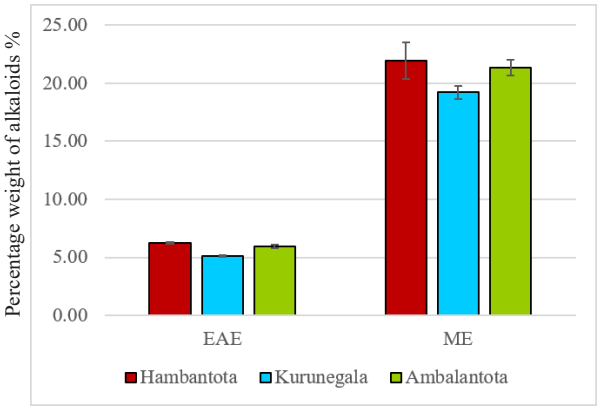

Figure 5: Comparison of alkaloid percentage.

The difference observed for the alkaloid percentage between the same extracts of different locations is less significant and almost lie within the error limits. Hamb-ME contains slightly higher amount of alkaloid content compared to Kuru-ME and Amba-ME. Alkaloid content in the hexane extracts, extracted by the acid-base method was

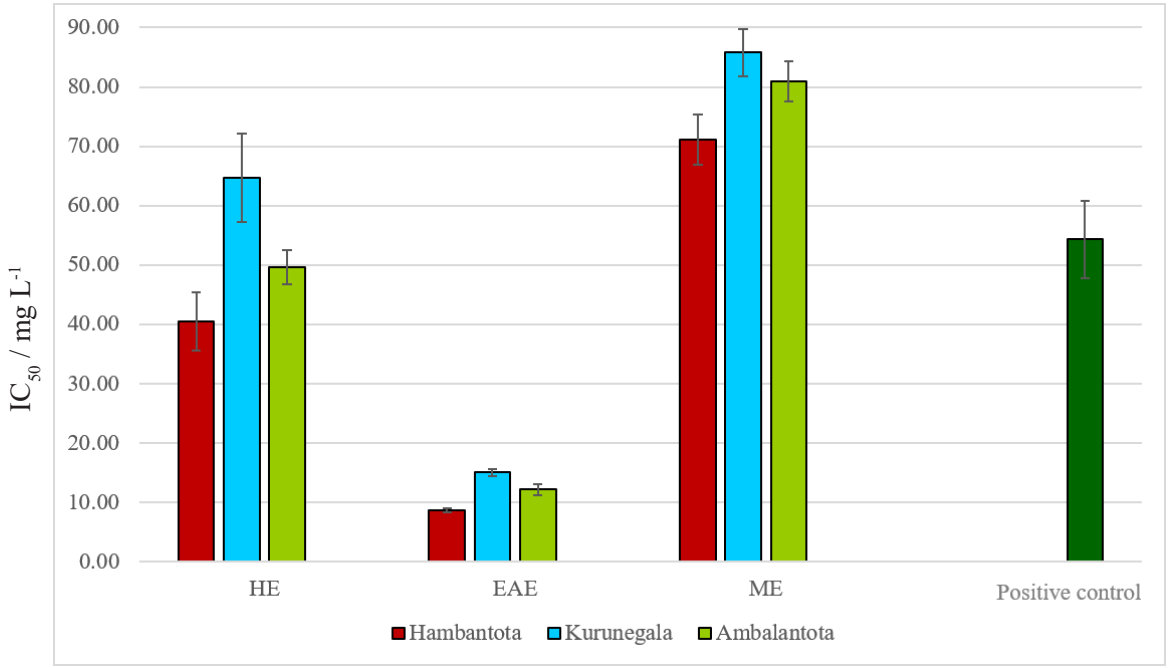

Figure 4: Comparison of LC_{50} values of brine shrimp lethality.

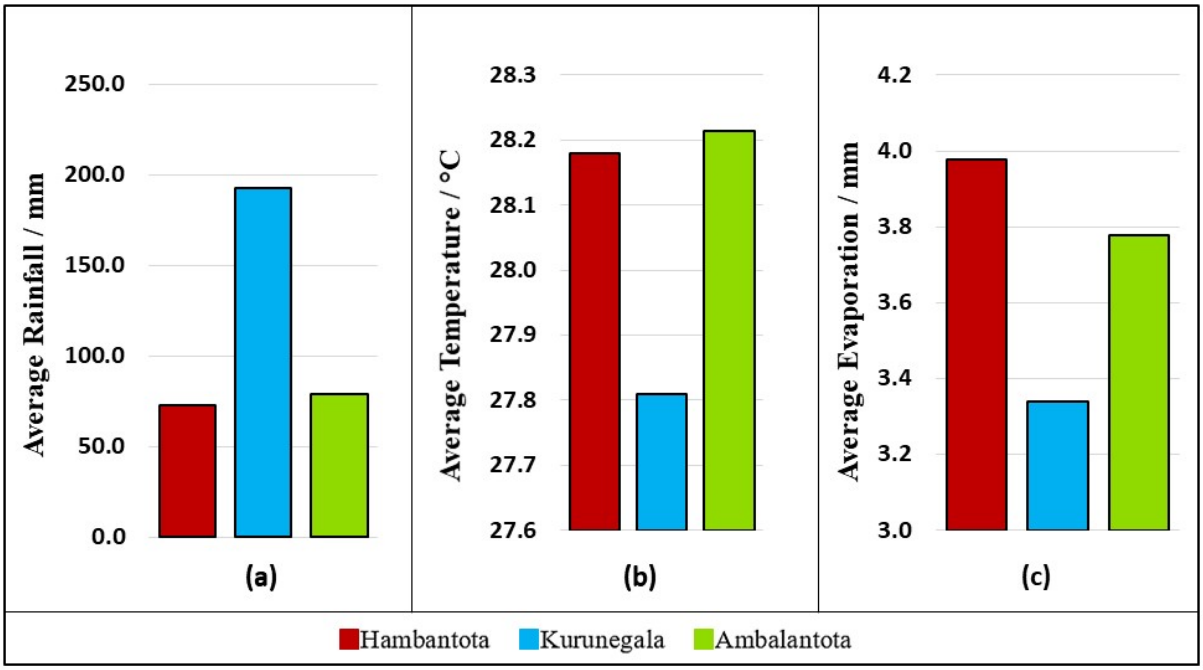

Figure 6: Analysis of climatic data (a) Average rainfall (b) Average Temperature (c) Average evaporation of Hambantota, Kurunegala and Ambalantota locations.

negligible, hence it is not discussed further.

When comparing the results of toxicity and alkaloid content of plant extracts, a proper correlation was not observed between the bioactivities. The MEs having the highest alkaloid percentage showed the lowest toxicity, while the EAEs showing the highest toxicity, showed the lower alkaloid percentage compared to MEs. Hence, it is clear that the observed toxicity is not only due to alkaloids present but is due to other groups of secondary metabolites as well.

Several studies reported the effect of pH of the substrate on which the plant grows to the production and the accumulation of secondary metabolites (Hager *et al*., 2008; Timsina *et al*., 2013; Radić, 2016). As a result, the bark pH of the host, *L. acidissima*, was measured at the region of the plant where the hemiparasite grew. However, a significant variation of the bark pH was not observed for the host plants of the three locations with a relatively neutral pH which varied from pH 6.26-7.14 (Table 2).

Table 2: Bark pH values of *L. acidissima*.

Location	Bark pH
Hambantota	6.26 ± 0.25
Kurunegala	7.14 ± 0.28
Ambalantota	6.88 ± 0.18

The bark pH of the host plant in Hambantota recorded the lowest pH (pH 6.26) and that of Kurunegala recorded the highest (pH 7.14). This suggests that the bark pH may have exerted a relatively similar effect in governing the production of secondary metabolites on *D. falcata* in all three locations.

When analyzing the variation of climatic parameters throughout 2017 and 2018 (Figure 6), it is clear that Hambantota has experienced more environmental stress compared to the other two locations. Kurunegala and Ambalantota have experienced the least and intermediate environmental stress respectively. The average rainfall was highest for Kurunegala (193 mm) while both Ambalantota (79 mm) and Hambantota (73 mm) locations have experienced relatively similar rainfall. Average evaporation was highest in Hambantota (4 mm) compared to Ambalantota (3.8 mm) and Kurunegala (3.3 mm). The average temperatures observed in all three locations are similar. As per the previous studies, harsh environmental conditions can boost the production of secondary metabolites in plant bodies. (Ramakrishna *et al*., 2011; Medina *et al*., 2014). Low rainfall together with high evaporation depicts the relatively harsh environmental conditions of Hambantota compared to the other two locations. When the environmental stress and the bioactivities observed for the extracts of the hemiparasite grown in particular bioclimatic zones are compared, a correlation is observed for some bioactivities while it is less significant for the others. The polyphenolic content shows a significant difference in activity which aligns well with the hypothesis of harsh environmental conditions cause greater production and accumulation of secondary metabolites that will help the survival of the plant. Toxicity results too correlate with the environmental stress conditions of bioclimatic zones of which samples were collected. Plant material collected from dry zone tends to have more toxic substances than that from the intermediate zone. The antioxidant activity is not inclined towards the hypothesis while the difference

in alkaloid content is less significant though Hambantota extracts showed slightly higher values.

CONCLUSION

In comparison of the extracts of the three locations selected for the study, Hambantota extracts showed the highest activity against almost all the bioassays conducted with significant differences for some bioactivities which was followed by Ambalantota and Kurunegala extracts respectively. The background data observed indicate that Hambantota and Ambalantota belonging to the dry bioclimatic zone experience more environmental stress compared to the intermediate bioclimatic zone to which Kurunegala belongs. Since the host plant is the same for all three locations, one of the main factors that determine the variation of bioactivity of the hemiparasite is the environmental stress they undergo. Because that will trigger the production and accumulation of secondary metabolites as a survival response by the plant, in turn resulting in higher bioactivity. The results of this study clearly suggest that the environmental conditions have an impact in governing the production of secondary metabolites in *D. falcata* on the host *L. acidissima* and hence a difference in bioactivity with a positive correlation with the environmental stress.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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